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Conservation of the response regulator gene *gacA* in *Pseudomonas* species

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Summary

The response regulator gene *gacA* influences the production of several secondary metabolites in both pathogenic and beneficial *Pseudomonas* spp. In this study, we developed primers and a probe for the *gacA* gene of *Pseudomonas* species and sequenced a 425 bp fragment of *gacA* from ten *Pseudomonas* strains isolated from different plant-associated environments. Polymerase chain reaction analysis and Southern hybridization showed that *gacA* is highly conserved within the genus *Pseudomonas*: multiple strains of different *Pseudomonas* species all responded positively to the probe, whereas no response was obtained from 18 other strains representing 14 species that belong to eight different genera of Gram-negative bacteria other than *Pseudomonas*. Furthermore, from a total of approximately 550 indigenous bacterial isolates obtained from the rhizosphere of wheat, all isolates that hybridized with the gacA probe were classified as *Pseudomonas* spp. by group-specific primers. Isolates that did not respond with the gacA probe and primers were identified as bacterial genera other than *Pseudomonas*, including *Stenotrophomonas*, *Cryseomonas* and *Comamonas* spp. These results indicate that *gacA* can be used as a complementary genetic marker for detection of *Pseudomonas* spp. in environmental samples. Phylogenetic relationships inferred from the newly sequenced *gacA* fragments and the sequences of *gacA* homologues present in the databases, showed six distinct clusters that correspond to the following bacterial families: Pseudomonaceae, Enterobacteriaceae, Alteromonadaceae, Vibrionaceae, Burkholderia and Xanthomonas. Within the Pseudomonadaceae and Enterobacteriaceae, polymorphisms within *gacA* and its homologues allowed identification of six and five subclusters respectively. Comparison of the *gacA* gene and GacA protein-based trees with the tree inferred from 16S rDNA sequences yielded a similar overall clustering. These results suggest that *gacA* and its homologues may provide complementary markers for phylogenetic studies of *Pseudomonas* spp. and Gram-negative bacteria other than *Pseudomonas*.

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Introduction

Bacteria of the genus *Pseudomonas* are able to survive and prosper in a wide range of environmental conditions. This genus not only contains plant, animal and human pathogenic species, but also accomodates species that are of significant environmental importance, including plant growth promoters, xenobiotic degraders, and biocontrol agents (O'Sullivan and O'Gara, 1992; Palleroni, 1992; Johnsen *et al*., 1996). Their versatile metabolic activities and ability to produce a wide variety of secondary metabolites have stimulated numerous ecological, molecular and biochemical studies.

In several strains that belong to the group of plant-associated *Pseudomonas* species, expression of genes involved in the biosynthesis of secondary metabolites and extracellular enzymes is positively controlled by the GacS/GacA two-component system (reviewed by Heeb and Haas, 2001). This regulatory system consists of a membrane-bound sensor kinase protein (GacS) and a cytoplasmic response regulator protein (GacA). The current model proposes that GacS recognizes specific environmental stimuli and activates GacA which in turn triggers the expression of specific genes (Appleby *et al*., 1996; Heeb and Haas, 2001; Pernestig *et al*., 2001). Several other elements are also involved in the regulation of certain genes, forming a complex regulatory cascade, with the GacS/GacA pair controlling secondary metabolite production at a higher hierarchy (Sarniguet *et al*., 1995; Whistler *et al*., 1998; Chancey *et al*., 1999; Blumer and Haas, 2000). For example, production of phenazine antibiotics, which are involved in the biocontrol activity and ecological competence of several *Pseudomonas* strains

(Mazzola *et al*., 1992), is partially regulated by the PhzI/PhzR quorum sensing system that uses *N*-acyl-homoserine lactone (Pierson *et al*., 1994). The GacS/GacA system interacts positively with the PhzI/PhzR quorum sensing system by regulating the synthesis of *N*-acyl-homoserine lactone thereby controlling phenazine production at the transcriptional level (Chancey *et al*., 1999).

The sensor kinase gene *gacS* was first described in *Pseudomonas syringae* pv. *syringae* as an essential factor for disease manifestation (Hrabak and Willis, 1992). Since then, *gacS* homologues have been identified in other *Pseudomonas* strains, *Azotobacter vinelandii*, *Vibrio cholerae*, and several enteric bacteria including *Escherichia coli*, *Salmonella enterica* serovar *typhimurium* and *Erwinia carotovora* ssp. *carotovora* (Heeb and Haas, 2001). Alignment of GacS homologues resulted in three families: the pseudomonads, the enteric bacteria and *Vibrio/Shewanella* (Heeb and Haas, 2001). Interestingly, within a total of nine *Pseudomonas* strains included in their analyses, *P. fluorescens, P. chlororaphis, P. tolaasii, P. viridiflava* and *P. syringae* belonged to a subgroup, whereas GacS homologues from *P. aeruginosa* and *P. putida* clustered at some distance (Heeb and Haas, 2001).

The response regulator gene *gacA (*global activator of cyanide and antibiotic production) was first identified in the biocontrol strain *P. fluorescens* CHA0 and was shown to be essential for the biocontrol activity and ecological fitness of this strain (Laville *et al*., 1992; Natsch *et al*., 1994). Homologues of GacA have been identified in several other Gram-negative bacterial genera. Within the family Pseudomonadaceae, Heeb and Haas (2001) described GacA homologues in a total of eight *Pseudomonas* strains, including *P. aeruginosa, P. viridiflava, P. syringae* pv. *syringae, P. fluorescens* and *P. aureofaciens*. Subsequent alignment of the GacA proteins placed these eight *Pseudomonas* strains at some distance from GacA homologues of enteric bacteria, and *Xylella* and *Shewanella*. The results from these *in silico* analyses suggested that the *gacA* gene may be conserved within Pseudomonadaceae. However, most information on *gacS*/*gacA* regulatory systems stems from studies on model strains and little information is available on the distribution of these genes in populations of rhizosphere-associated bacteria.

In this study, we developed primers and a probe for the *gacA* gene of *Pseudomonas* species and sequenced a 425 bp fragment of the *gacA* gene from ten *Pseudomonas* strains isolated from different plant-associated environments. The newly sequenced *gacA* fragments as well as *gacA* homologues currently present in databases were used in phylogenetic analyses. Delineation of *Pseudomonas* species based on *gacA* gene and protein sequences was compared to classification based on 16S rDNA sequences. Sequence data were also used to compare the rate of evolution of the *gacA* gene as well as the ratio of synonymous to non-synonymous substitutions in *gacA* sequences within and between Pseudomonaceae and Enterobacteriaceae. The potential usefulness of the *gacA* gene as a complementary genetic marker for *Pseudomonas* was evaluated on bacterial populations isolated from the rhizosphere of wheat. Approximately 550 isolates were genotypically and phenotypically identified and characterized for the production of specific extracellular enzymes and secondary metabolites known to be regulated by GacA.

Results

Design and evaluation of the gacA *primers and probe*

Primers gacA1 (5′-GBATCGGMGGYCTBGARGC-3′) and gacA2 (5′-MGYCARYTCVACRTCRCTGSTGAT-3′) were developed from conserved sequences within the *gacA* genes of *P. fluorescens* strains BL915 (GenBank L29642), CHA0 (GenBank M80913), Pf-5 (GenBank U30858), *P. aeruginosa* PAO1 (GenBank U27988), *P. syringae* pv. *syringae* B728a (GenBank U09767), *P. chlororaphis* 30–84 (GenBank AF115381) and the *repB* gene of *P. viridiflava* PJ-08–6 A (GenBank L30102). Primers gacA1 and gacA2 are complementary to positions 489 and 914, respectively, in the *gacA* sequence of *P. fluorescens* strain CHA0. Primers gacA1 and gacA2 amplified the predicted 425 bp fragment of homologous strain *P. fluorescens* CHA0. Restriction analysis of the amplified fragment with enzymes *Ava*II, *Alu*I, *Fsp*I, *Hin*fI, *Nae*I and *Sty*I resulted in restriction fragments with sizes identical to those deduced from the restriction map of the *gacA* sequence of CHA0. The gacA primers amplified a 425 bp fragment from all *Pseudomonas* strains, representing both fluorescent and non-fluorescent species, except from *P. agarici* DSM11810, *P. fragi* DSMZ3456 and *P. fluorescens* strains 2–79RN10 and SS101 (Table 1). The PCR products obtained from all positive strains hybridized under high stringent conditions with the gacA probe consisting of the labelled 425 bp PCR fragment obtained from strain CHA0. In Southern hybridization with *Eco*RI-digested genomic DNA, all *Pseudomonas* strains tested, including DSM11810, DSMZ3456, 2–79RN10, and SS101, hybridized under high-stringent conditions with the gacA probe. A single hybridized band was detected for all strains. No PCR products or hybridization signals were obtained from 18 other strains, representing 14 species belonging to eight genera of Gram-negative bacteria other than *Pseudomonas* (Table 1).

Table 1. Specificity of the *gacA* primers and probe.

Species	Strain	Metabolites[a]	Specificity[b]	
			PCR	Hybrid
Pseudomonas aeruginosa	PAO1	RHL, pro	+	+
	DSM1128	RHL, pro	+	+
	DSM939	RHL	+	+
P. agarici	DSM11810		–	+
P. aureofaciens	LMD82.53	PRN, PHZ, pro, phc	+	+
P. fluorescens	CHA0	2,4-DAPG, PRN, PLT, pro, phc	+	+
	Pf-5	2,4-DAPG, PRN, PLT, pro, phc	+	+
	PGNR1	2,4-DAPG, PRN, PLT, pro, phc	+	+
	PINR2	2,4-DAPG, PRN, PLT, pro, phc	+	+
	F113	2,4-DAPG, pro	+	+
	Q8r1–96	2,4-DAPG, pro	+	+
	PILH1	2,4-DAPG, pro	+	+
	2–79RN10	PHZ, pro, phc	–	+
	SSB33		+	+
	Q2-87	2,4-DAPG	+	+
	SSB17	2,4-DAPG	+	+
	SS101	pro, phc	–	+
P. chlororaphis	PHZ50	PHZ, pro	+	+
	PHZ26	PHZ, pro, phc	+	+
Pseudomonas sp.	PHZ13	PHZ	+	+
P. syringae pv. *pisi*	24.7	pro	+	+
P. stutzeri	ATCC17588		+	+
P. mendocina	DSMZ50017		+	+
P. fragi	DSMZ3456		–	+
P. brassicacearum	DSMZ13227		+	+
P. putida	WCS358R		+	+
Burkholderia cepacia	LMG1222		–	–
	ATCC51671	PRN, pro, phc	–	–
	J2535	PRN, pro, phc	–	–
	J2540	PRN, pro	–	–
	J2553	pro, phc	–	–
B. pyrrocinia	DSM10685	PRN, pro	–	–
B. multivorans	LMG13010	phc	–	–
B. vietnamiensis	LMG10929	phc	–	–
Escherichia coli	JM107		–	–
Stenotrophomonas maltophilia	DSM8573	pro	–	–
St. nitritireducens	DSM12575		–	–
Xanthomonas fragariae	DSM3587		–	–
X. campestris pv. *campestris*	DSM 3586	pro	–	–
Flavobacterium aquatile	DSM1132	pro	–	–
Pantoea agglomerans	DSM8570		–	–
Serratia marcescens	DSM30121	pro, phc	–	–
S. plymuthica	DSM8571	pro, phc	–	–
Rhizobium radiobacter	DSM30205		–	–

a. The metabolites 2,4-DAPG (2,4-diacetylphloroglucinol) and PHZ (phenazines) were detected by PCR with specific primers (37), PRN (pyrrolnitrin) and PLT (pyoluteorin) were detected as described previously (Duffy and Défago, 1999, Keel *et al*., 1996), RHL (rhamnolipids) were detected by transferring colonies onto SW medium (Siegmund and Wagner, 1999), pro (protease) and phc (phospholipase C) were detected by plating colonies on skim milk medium and egg yolk plates respectively (Sacherer *et al*., 1994).
b. + : amplification of the predicted 425 bp fragment in PCR with primers gacA1 and gacA2. Hybridization under high or low stringent conditions with the 425 bp GacA probe derived from *P. fluorescens* CHA0; – : no amplification or hybridization with the gacA primers or probe respectively.

Sequence analysis of gacA *and 16S-rDNA*

Ten *Pseudomonas* strains were selected for sequencing of the *gacA* gene. The ten strains were *P. fluorescens* strains Q2-87, SSB17, F113, and SSB33; *P. chlororaphis* LMD 82.53 and PHZ26, *Pseudomonas* sp. PHZ13; *P. putida* WCS358R; *P. aeruginosa* DSM 1128, and *P. syringae* pv. *pisi* 24.7. The overall base composition of the newly obtained *gacA* sequences was $T = 20.4\%$ (s.d. $\pm$ 0.5), $C = 31.3\%$ ($\pm$ 0.6), $A = 21.0\%$ ($\pm$ 0.3), and $G = 27.4\%$ ($\pm$ 0.4). The identity of the new *gacA* sequences to the *gacA* sequence from strain CHA0 ranged from 79.6 to 89.2% and the similarity of the predicted GacA protein ranged from 85.9 to 98.5%. The 16S rDNA sequences were determined over a total of approximately 300–600 bp to verify or identify the following strains: *P. fluorescens* Pf-5, SSB33, and SSB17, *P. aeruginosa* DSM 1128, *P. chlororaphis* LMD 82.53, and PHZ26, *Pseudomo-*

nas sp. PHZ13, *P. putida* WCS358R and *P. syringae* pv. *pisi* 24.7. The sequence identity of the 16S rDNA in relation to that of strain CHA0 was 64.2% (399 nucleotides overlap) for strain SSB17, 73.5% (328 nucleotides overlap) for PHZ13, 81.5% (336 nucleotides overlap) for PHZ26, 82.3% (322 nucleotides overlap) for WCS358R, 83.3% (270 nucleotides overlap) for LMD 82.53, 87.3% (378 nucleotides overlap) for SSB33, 87.4% (496 nucleotides overlap) for Pf-5 and 88.1% (503 nucleotides overlap) for *P. syringae* pv. *pisi* 24.7.

Table 2. Accession numbers of *gacA* and 16S rDNA sequences.

		Accession number[a]	
Species	Strain	*gacA*	16S rDNA
Pseudomonaceae			
Azotobacter vinelandii	ATCC 9046	AF382827	–
Pseudomonas sp.	PHZ13	AF502416	AF495869
Pseudomonas aeruginosa	PAO1	U27988	AE004091
	DSM1128	AF502413	AF502423
P. aureofaciens	30–84	AF115381	–
P. chlororaphis	LMD82.53	AF502415	AF495868
	PHZ26	AF502417	AF502424
P. fluorescens	CHA0	M80913	AJ278812
	Pf-5	AF065156	AF495871
	F113	AF502414	AJ278814
	Q2-87	AF502419	AJ278813
	SSB33	AF502420	AF502427
	BL915	L29642	–
	SSB17	AF502421	AF502426
	PfO-1	Unfinished	Unfinished
	FPT9601	AB054363	–
P. putida	KT2440	AE016789	AE016791
	WCS358R	AF502422	AF502428
P. syringae pv. *pisi*	24.7	AF502418	AF502425
P. syringae pv. *syringae*	B728a	U09767	–
P. syringae pv. *Tomato*	DC3000	Unfinished	Unfinished
P. viridiflava	PJ-08–6 A	L30102	–
Enterobacteriaceae			
Escherichia coli	K-12 MG1655	M24615	AE000129
	RIMD0509952	AP002559	AP002566
	O157 EDL933	AE005414	AE005195
	CFT073	Unfinished	Unfinished
Klebsiella pneumoniae	MGH 78578	Unfinished	Unfinished
Pectobacterium caratovorum	SSC3193	X95564	
Salmonella typhi	CT18	AL627272	AL627282
	TY2	Unfinished	Unfinished
S. typhimurium	LT2	AE008786	AE008706
	ATCC 14028	U67869	
Shigella flexneri	2a	NC_004337	NC_004337
Yersinia pestis	CO-92	AJ414150	AJ414156
	KIM5 P12	NC_004088	NC_004088
Y. enterolitica	0:8	Unfinished	Unfinished
Alteromonadaceae			
Shewanella oneidensis	MR-1	AE015629	NC_004347
Vibrionaceae			
Vibrio cholerae	O395	AF071105	
	N16961	AE004201	AE004157
Xanthomonas group			
Xylella fastidiosa	9a5c	AE004068	AE003870
	Ann-1	NZ_AAAM01000019	NZ_AAAM01000002
	Dixon	NZ_AAAL010000114	NZ_AAAL01000059
	Temecula 1	Unfinished	Unfinished
Burkholderia group			
Burkholderia cepacia	J2315	Unfinished	Unfinished

a. Unfinished refers to non-annotated data obtained from genome sequencing projects. (–) refers to absence of data on these strains in the databases.

Phylogenetic analyses

The neighbour-joining tree inferred from the newly sequenced *gacA* genes plus the sequences of *gacA* homologues already present in the databases (Table 2), showed six distinct clusters that correspond to the following bacterial families: Pseudomonaceae (I), Enterobacteriaceae (II), Vibrionaceae (III), Alteromonadaceae (IV), Burkholderia (V), and Xanthomonas (VI) (Fig. 1A). Within the Pseudomonadaceae, six subclusters can be distinguished based on significant bootstrap values (>80%): the first contained *Pseudomonas* sp. PHZ13, *P. chlororaphis*, *P. aureofaciens*, and *P. fluorescens* CHA0, Pf-5, and BL915; the second subcluster contained only *P. fluorescens* strains; the third contained *P. viridiflava* and *P. syringae* strains; the fourth was composed of two *P. putida* strains, the fifth of *P. aeruginosa* strains, and the sixth of *Azotobacter vinelandii*. Within the enteric bacteria, five subclusters were distinguished: the first one comprised *E. coli* isolates and *Shigella flexneri*, which were tightly clustered; the second contained only *Salmonella* species; the third subcluster contained *Klebsiella pneumoniae*; the fourth, the plant pathogenic *Pectobacterium caratovorum*, and the fifth contained *Yersinia* species (Fig. 1A). Other major clusters contained the Vibrionaceae, represented by *Vibrio (*cluster III), Alteromonadaceae, represented by *Shewanella putrefaciens* (cluster IV), the Burkholderia family, represented by *B. cepacia* (cluster V), and the Xanthomonas family, represented by *Xylella fastidiosa* isolates (cluster VI). Cluster VI was the most distant of all clusters obtained (Fig. 1A). In all cases, with exception of clusters V and VI, the bootstrap values were highly significant. Similar results were obtained with the neighbour-joining tree inferred from the predicted GacA protein sequences (data not shown), although the length of the branches defining clusters were shorter than those in the

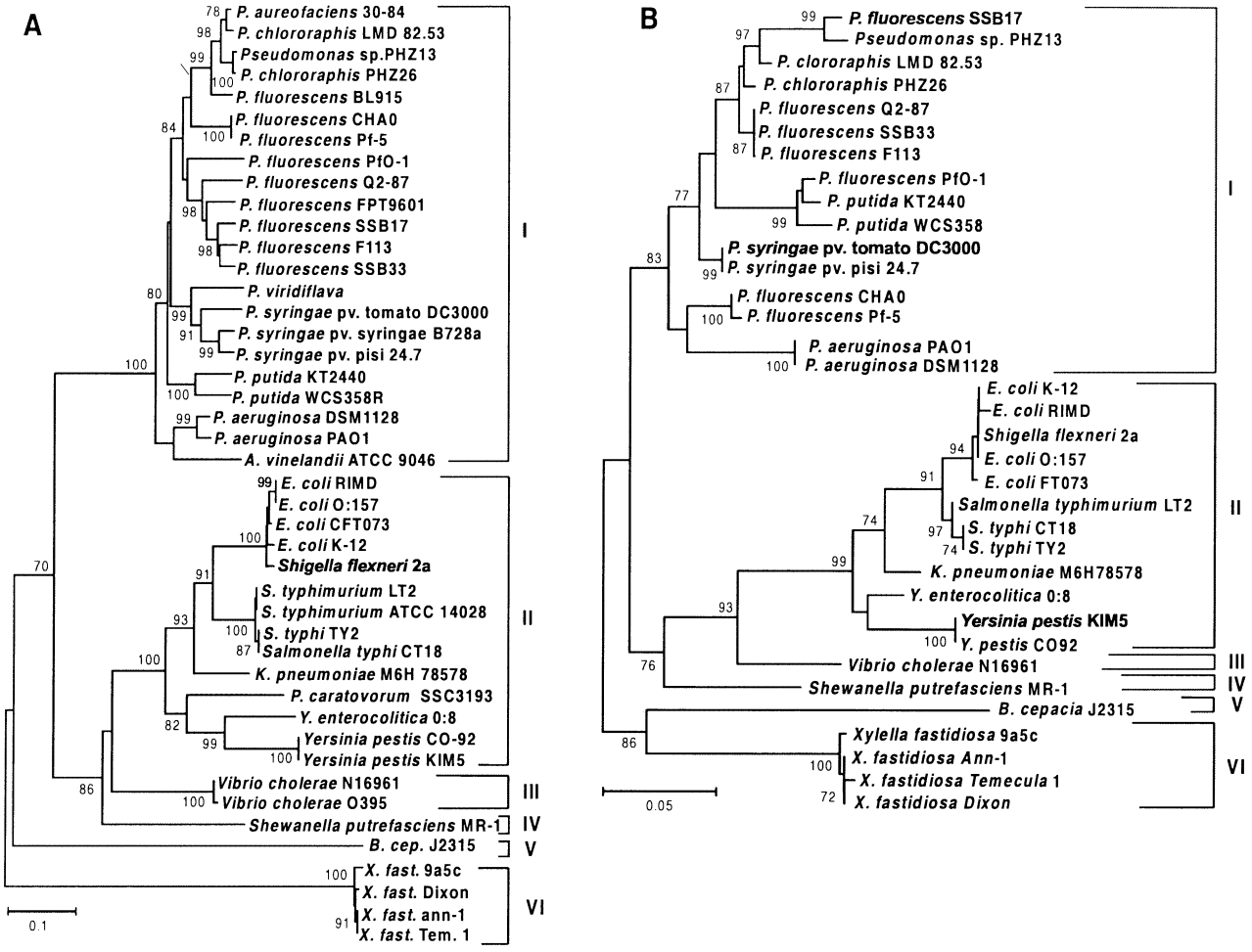

Fig. 1. Phylogenetic trees inferred by the neighbour-joining method with sequences of *gacA* (A), and 16S rDNA (B).
A. Based on 406 bp aligned *gacA* nucleotides (positions 501–906 of the *gacA* sequence of *P. fluorescens* CHA0).
B. Based on 270 bp aligned 16S rDNA sequences (positions 1–270 of the nucleotide sequence of *P. fluorescens* CHA0). Bootstrap values greater than 70% are shown.

Table 3. Non-synonymous (Ka) and synonymous (Ks) substitution rates in *gacA* sequences.

Categories[a]	N[b]	Ka	Ks	Ka/Ks
All sequences	44	0.304 ± 0.017	0.437 ± 0.029	0.696
Pseudomonaceae and Enterobacteriaceae	36	0.165 ± 0.017	0.643 ± 0.018	0.257
Pseudomonaceae	22	0.034 ± 0.001	0.519 ± 0.009	0.063
Enterobacteriaceae	14	0.055 ± 0.004	0.582 ± 0.029	0.074

a. Comparisons of the *gacA* sequences in different categories. The categories of the *gacA* gene and its homologues were: (1) all *gacA* sequences together; (2) *gacA* sequences from the Pseudomonadaceae and Enterobacteriaceae; (3) only *gacA* sequences from Pseudomonadaceae; and (4) only *gacA* sequences from Enterobacteriaceae.
b. Number of sequences per category.

tree inferred from the *gacA* gene sequences. Comparison of the *gacA* gene-based tree with the tree inferred from 16S rDNA sequences yields a similar overall clustering (Fig. 1B), although some incongruences were noted. In the 16S rDNA-based tree, *P. fluorescens* SSB17 clustered closer to *P. chlororaphis* and *P. aureofaciens* than to other *P. fluorescens* strains. Furthermore, *P. fluorescens* strains CHA0 and Pf-5 were clustered closer to *P. aeruginosa*. The 16S rDNA clustering of *B. cepacia* was in agreement with the clustering based on *gacA* gene sequences (Fig. 1A). Phylogenetic analyses of the *gacA* gene, predicted GacA protein and 16S rDNA sequences with the maximum likelihood method yielded similar results as obtained by neighbour-joining.

Rate of evolution of gacA

The measure of non-synonymous relative to synonymous substitution rates (Ka/Ks) provides insight into the level of selective constraint acting on proteins (Li *et al*., 1985). Protein-encoding genes evolving at a neutral rate have Ka/Ks ratios equal to 1, indicating that the protein evolves without constraint on amino acid replacements. When Ka/Ks < 1, the protein is under selective constraints and protein-encoding genes are subjected to purifying selection. Finally, when Ka/Ks > 1, positive selection has acted to promote amino acid replacements (Li *et al*., 1985; Hughes and Nei, 1988). The Ka/Ks ratio for *gacA* genes was determined for different categories and was shown always to be smaller than 1 (Table 3). Fisher's exact test ($P = 0.05$) and *Z*-test confirmed that purifying selection is operating on the *gacA* genes of both Pseudomonadaceae and Enterobacteriaceae.

Tajima's relative rate test was performed to test the molecular clock hypothesis. According to this hypothesis, sequences evolve at the same rate, irrespective of the substitution model and whether or not the substitution rate varies with site (Tajima, 1993). The test was done in 45 pairwise combinations with a third *gacA* sequence as outgroup. Before this analysis, majority sequences of *gacA* of species belonging to the Pseudomonaceae and Enterobacteriaceae were computed to reduce the number of two-by-two combinations. Tajima's test showed that there were no statistically significant differences in every possible pairwise comparison between members of the Pseudomonaceae, with *K. pneumoniae* as outgroup. Comparisons of members of the Pseudomonaceae with members of the Enterobacteriaceae, with *B. cepacia* as outgroup, showed that 13 out of 24 possible combinations gave statistically different evolutionary rates. In all these 13 statistically different combinations, members of the Enterobacteriaceae presented significantly higher substitution rates than members of the Pseudomonaceae (i.e. $m_2 > m_1$). Within the Enterobacteriaceae, with *A. vinelandii* as outgroup, five out of ten possible pairwise comparisons showed significantly different substitution rates. Collectively, these results indicate that all tested *gacA* sequences from Pseudomonadaceae evolve at the same rate. In contrast, approximately 50% of the comparisons within the Enterobacteriaceae, and between Enterobacteriaceae and Pseudomonaceae gave significantly different substitution rates, suggesting higher evolutionary rates for the *gacA* gene in the Enterobacteriaceae.

Evaluation of gacA *as a complementary genetic marker for* Pseudomonas *spp.*

Populations of indigenous bacteria harbouring homologues of *gacA* found in *Pseudomonas* spp. were isolated from roots of wheat by colony hybridization with the gacA probe and subsequent PCR with the gacA primers. On KMB^+, a medium semi-selective for *Pseudomonas* (Simon and Ridge, 1974), their densities ranged from approximately 5.0×10^6–1.8×10^7 cfu g^{-1} root and represented on average 35.8% to 73.5% of the total bacterial population recovered on this medium (Table 4). Based on colony hybridization and subsequent PCR, 494 *gacA* positive (referred to as '*gacA*$^+$') and 58 *gacA* negative (referred to

Table 4. Frequency of bacterial isolates from the rhizosphere of wheat, grown in different soils, that responded positively to the gacA probe ('*gacA*$^+$') derived *from P. fluorescens* strain CHA0.

Soil	Total population[a] (cfu g^{-1} root)	'*gacA*$^+$' [b] (cfu g^{-1} root)	'*gacA*$^+$'/total (%)
CB	1.9×10^7	9.8×10^6	52.4
SL	1.5×10^7	5.4×10^6	35.8
SU	2.9×10^7	1.5×10^7	51.0
SV	2.5×10^7	1.8 X107	73.5
SSB	2.4×10^7	1.7×10^6	69.4

a. Total population of bacteria isolated on King's medium B.
b. Colony hybridization followed by PCR was used to determine the frequencies of isolates that responded positively to the gacA probe and gacA primers (referred to as '*gacA*$^+$'). Mean values of four replicates are shown.

Table 5. Phenotypic and genotypic characteristics of bacterial isolates obtained from the wheat rhizosphere that responded positively ('*gacA*$^{+}$') or negatively ('*gacA*$^{-}$') to the gacA probe derived from *P. fluorescens* strain CHA0.

Analysis	'*gacA*$^{+}$'[a]	'*gacA*$^{-}$'[a]
Number of isolates	494	58
Unique RAPD groups[b]	116	34
2,4-diacetylphloroglucinol[c]	33.8%	0.0%
Phenazines[c]	3.0%	0.0%
Fluorescence[c]	88.0%	0.0%
Protease[c]	71.0%	69.0%
Phospholipase C[c]	19.6%	70.7%
Surfactants[c]	0.6%	0.0%
Chitinase	0.0%	55.2%
Identity[d]	*Pseudomonas* (100%)	*Stenotrophomonas* (62%), *Comamonas* (14%), *Cryseomonas* (5%), unidentified (19%)

a. Percentage in relation to the number of isolates.
b. RAPD analysis was performed with three 10-mer primers and 100% similarity was used to define a unique group.
c. Metabolites were detected as mentioned in Table 1.
d. Identity determined with *Pseudomonas* group-specific primers targeting 16S rDNA (Widmer *et al*., 1998), API 20NE test and GC-FAME analysis.

as '*gacA*$^{-}$') isolates were selected for further genotypic and phenotypic analyses (Table 5). Random Amplified Polymorphic DNA (RAPD) analysis with three 10-mer primers showed 116 and 34 unique groups among the '*gacA*$^{+}$' and '*gacA*$^{-}$' isolates respectively. Characterization of representatives from all RAPD groups, employing Southern hybridization of *EcoR*I-digested genomic DNA, confirmed that all '*gacA*$^{+}$' isolates responded positively with the gacA probe, whereas all 58 '*gacA*$^{-}$' isolates responded negatively, even at low-stringent conditions (data not shown). Based on PCR with group-specific primers (Widmer *et al*., 1998), all '*gacA*$^{+}$' isolates were identified as *Pseudomonas*, whereas none of the 58 '*gacA*$^{-}$' isolates gave a positive response. Based on GC-FAME analysis of the '*gacA*$^{-}$' isolates, 62% were classified as *Stenotrophomonas maltophilia*, 14% as *Comamonas acidovorans*, 5% as *Cryseomonas indologenes* and 19% could not be identified conclusively due to low matching coefficients.

Subsequent characterization of the isolates for the production of specific secondary metabolites and extracellular enzymes, known to be regulated by *gacA,* showed that none of the '*gacA*$^{-}$' isolates displayed fluorescence, produced surfactants or harboured biosynthetic genes for 2,4-diacetylphloroglucinol and phenazine antibiotics (Table 5). Fluorescence and surfactant production was found in 88% and 0.6% of the '*gacA*$^{+}$' isolates, respectively, and genes for 2,4-diacetylphloroglucinol (2,4-DAPG) and phenazine antibiotics were found in 34% and 3% of the '*gacA*$^{+}$' isolates respectively. Similar frequencies of 2,4-DAPG and phenazine producing *Pseudomonas* spp. have been reported for the wheat rhizosphere in previous studies (Raaijmakers *et al*., 1997; de Souza *et al*., 2003). Both '*gacA*$^{+}$' and '*gacA*$^{-}$' isolates produced protease and phospholipase C, enzymes that are under the control of *gacA* in the biocontrol strain *P. fluorescens* CHA0 (Sacherer *et al*., 1994). Protease production was found in similar proportions of '*gacA*$^{+}$' and '*gacA*$^{-}$' isolates *(*~70%), whereas phospholipase C was detected in a higher proportion of the '*gacA*$^{-}$' isolates. Chitinase was detected only in '*gacA*$^{-}$' isolates and in particular in the isolates that were identified by GC-FAME analysis as *Stenotrophomonas maltophilia*.

Discussion

The response regulator gene *gacA* influences the production of several secondary metabolites in both pathogenic and beneficial *Pseudomonas* spp. (Gaffney *et al*., 1994; Heeb and Haas, 2001). In this study, we demonstrated that *gacA* is highly conserved within the genus *Pseudomonas*. In Southern hybridization with the gacA probe, multiple strains of different *Pseudomonas* species all responded positively, whereas no response was obtained from 18 other strains representing 14 species belonging to eight genera of Gram-negative bacteria other than *Pseudomonas* (Table 1). Furthermore, from a total of approximately 550 indigenous bacterial isolates obtained from the rhizosphere of wheat, all isolates that responded positively to the gacA probe and primers (referred to as *gacA*$^{+}$) were classified as *Pseudomonas* spp., whereas isolates that did not hybridize with the gacA probe (referred to as *gacA*$^{-}$) were identified as bacterial genera other than *Pseudomonas*, including *Stenotrophomonas*, *Cryseomonas* and *Comamonas* spp. (Table 5). Based on RAPD analyses, both *gacA*$^{+}$ and *gacA*$^{-}$ isolates represented a large number of genotypically different groups. These results indicate that *gacA* can be used as a complementary genetic marker for detection of *Pseudomonas* spp. in rhizosphere samples. They also show that the

selectivity of King's medium B for *Pseudomonas* spp. is limited, although all fluorescent bacteria recovered on this medium responded positively to hybridization and PCR with the gacA probe and primers, and were identified as *Pseudomonas* spp. by group-specific primers.

Although many studies have focused on the genus *Pseudomonas*, their taxonomy and phylogenetic relationships are far from being completely resolved (Bossis *et al*., 2000). Among the various methods used to identify and classify pseudomonads, DNA–DNA hybridization and 16S rDNA sequencing are the most advocated ones (Wayne *et al*., 1987; Anzai *et al*., 2000). The use of both methods, however, may present certain limitations. DNA–DNA homology has not been powerfull enough to reveal phylogenetic relationships (Anzai *et al*., 2000). Also, sequences of 16S rDNA present limited power when analysing closely related organisms that diverged at almost the same time (Woese, 1987; Fox *et al*., 1992). Furthermore, 16S rDNA sequences occur in multiple copies in a single cell and, interestingly, these copies were shown to evolve at different rates (Ueda *et al*., 1999).

In this study, phylogenetic relationships inferred from *gacA* sequences revealed six distinct bacterial families, including the Pseudomonaceae, Enterobacteriaceae, Alteromonadaceae, Vibrionaceae, Burkholderia, and Xanthomonadaceae (Fig. 1). Within the Pseudomonaceae, polymorphisms within the *gacA* gene allowed further distinction of at least six subclusters. Although similar phylogenetic relationships were found for 16S rDNA sequences, some differences were found. For example, *P. fluorescens* strains CHA0 and Pf-5 were clustered closer to *P. aeruginosa* and *P. syringae* based on 16S rDNA sequences, whereas clustering based on *gacA* gene and GacA protein sequences placed both strains closer to the other *P. fluorescens* strains. Discrepancies between phylogenies of *Pseudomonas* inferred from 16S rDNA and other genes were also observed for *gyrB* and *rpoD* (Yamamoto and Harayama, 1998; Yamamoto *et al*., 2000). One may argue that some of the discrepancies found in this study could be due to the relatively small size of the 16S rDNA sequences used. However, other phylogenetic studies have employed fragments of the same size as used in this study or even smaller (Borneman *et al*., 1996; Zhu *et al*., 2002). Furthermore, it has been shown that phylogenetic assignements were similar whether partial or full-length sequences of 16S rDNA were used in the analyses (Schmidt *et al*., 1991). Given the relatively small size of the *gacA* gene and its apparent occurrence as a single copy in *Pseudomonas* makes the *gacA* gene not only a useful complementary genetic marker for detection of *Pseudomonas* but also eligible for phylogenetic studies. It is remarkable that a small sized gene like *gacA* has such strong resolving capacity for phylogenetic purposes. Comparable results were obtained with the 249 bp *oprI* gene, which allowed molecular taxonomic studies in pseudomonads belonging to the rRNA group I, a group that contains the genuine *Pseudomonas* species (De Vos *et al*., 1998). Interestingly, polymorphisms within *gacA* homologues also allowed distinction of five subclusters in the family of enteric bacteria, including *E. coli*, *Salmonella, K. pneumoniae, P. caratovorum* and *Yersinia* species (Fig. 1). These results suggest that *gacA* homologues also may provide a complementary genetic marker for phylogenetic studies of Gram-negative bacteria other than *Pseudomonas*. More sequences of *gacA* homologues of representative strains of different enteric and other bacterial genera will be required to further support this hypothesis.

Estimations of non-synonymous to synonymous substitution rates (Ka/Ks) indicated that, for members of both the Pseudomonaceae and Enterobacteriaceae, purifying selection is acting on *gacA* (Table 3). These results indicate that there is selective pressure to avoid substitutions leading to functional changes in the GacA protein. In this context, it is interesting to note that for several *P. fluorescens* strains spontaneous mutations in *gacA* and also *gacS* have been described to occur frequently under laboratory conditions (Whistler *et al*., 1998; Duffy and Défago, 2000). In *P. fluorescens* strain CHA0, grown in nutrient-rich conditions, spontaneous mutants accumulated over a period of several days to levels of 1.25% of the population and half of the mutants tested were restored by *gacA* (Bull *et al*., 2001). Subsequent competition experiments showed that the *gacA* mutant had only a temporary selective advantage over the wild type that was restricted to an early phase of the stationary phase (Bull *et al*., 2001). The role of the *gacA/gacS* pair in surviving exposure to oxidative stress (Whistler *et al*., 1998), and in production of secondary metabolites that play a significant role in the ecological fitness of certain *Pseudomonas* strains (Mazzola *et al*., 1992) provide, at least in part, an explanation for the relatively low rate of non-synonomous substitutions in *gacA*. Tajima's relative rate test showed that in 50% of the comparisons between members of the Enterobacteriaceae and Pseudomonadaceae, evolutionary rates in *gacA* were significantly different. So, in several members of the Enterobacteriaceae the *gacA* gene appears to evolve faster than in members of the Pseudomonaceae, even though purifying selection is acting on *gacA* of both bacterial families. Comparative sequencing of environmental and clinical isolates of *P. aeruginosa* (Kiewitz and Tümmler, 2000) showed that the sequence diversity in multiple genes of *P. aeruginosa* was about one order of magnitude lower than in comparable housekeeping genes of *Salmonella.* One may speculate that many enteric bacterial strains have a parasitic lifestyle and a higher exposure to therapeutic antibiotics for which a higher adaptive capacity may be required. How-

ever, the parasitic life-style also applies to several plant and human pathogenic bacterial species of the Pseudomonaceae, including *P. syringae* and *P. aeruginosa*. More studies will be necessary to elucidate the biological relevance, if any, of the relatively higher evolutionary rates of *gacA* in several members of the Enterobacteriaceae as compared to Pseudomonaceae.

In conclusion, the results of this study show that the response regulator gene *gacA* is conserved in *Pseudomonas* species, is subjected to purifying selection, and can serve as a complementary genetic and phylogenetic marker for population biology of *Pseudomonas* in rhizosphere environments.

Experimental procedures

Bacterial strains and culture conditions

Naturally occurring *Pseudomonas* spp. were isolated from roots of wheat grown in natural soils on the semiselective King's medium B (KMB) agar supplemented with chloramphenicol (13 μg ml^{-1}), ampicillin (40 μg ml^{-1}), and cycloheximide (100 μg ml^{-1}) [KMB^{+}] (Simon and Ridge, 1974). Bacterial isolates were routinely grown on KMB medium. For DNA extractions, bacteria were cultivated overnight at 24°C on nutrient broth yeast extract agar (Vidaver, 1967). *Escherichia coli* strains were grown in LB (Luria–Bertani). All bacterial strains were stored at −80°C in LB or KMB broth supplemented with 40% glycerol (v/v).

DNA extractions

For all PCR analyses, including RAPD analysis, heat-lysed bacterial suspensions were used and prepared as described previously (Raaijmakers *et al*., 1997). Total DNA for use in PCR amplifications of the 16S rDNA genes was obtained as described by Raaijmakers *et al*. (1997). Total genomic DNA, used for Southern blot analysis and *gacA* amplifications, was extracted from bacterial strains by a modified version of a cetyltrimethylammoniumbromide (CTAB)-based protocol (Ausubel *et al*., 1992). A 1.5 ml sample of bacterial cultures grown overnight was centrifuged for 3 min at 14 000 r.p.m.; supernatant was discarded and the pellet was resuspended in 550 μl of TE buffer (Tris 10 mM, EDTA 10 mM, pH 8.0) amended with lysozyme (1.82 mg ml^{-1}); the mixture was incubated at 37°C for 30 min. Seventy-five μl of 10% SDS amended with proteinase K (0.86 mg ml^{-1}) was added to the bacterial suspension and thoroughly mixed. After 15 min incubation at 65°C, 100 μl of 5 M NaCl and 80 μl CTAB/NaCl (0.3 M CTAB, 0.7 M NaCl) were added. After 10 min of incubation at 65°C, DNA was obtained by extraction with chloroform/isoamyl alcohol (24:1, v/v), isopropanol precipitation and subsequent washes with 70% ethanol. The extracted DNA was dissolved in 100 μl of 10 mM Tris pH 8.0 containing RNAase (20 μg ml^{-1}) and stored at −20°C. Restriction analyses of genomic DNA were performed with 5 U of *Eco*RI (Promega), an enzyme without restriction sites in the *gacA* genes of *P. fluorescens* strains BL915, CHA0, and Pf-5, *P. aeruginosa* PAO1, *P. syringae* pv. *syringae* B728a, *P. chlororaphis* 30–84, and the *repB* gene of *P. viridiflava* PJ-08–6 A. Digestions were performed in a total volume of 100 μl containing 2.0 μg of DNA; digested DNA was precipitated with 4 M LiCl, washed with 70% ethanol, dissolved in 15 μl of sterile distilled water and separated on 1% agarose gels in TBE.

PCR amplification of the gacA *gene*

Primers gacA1 and gacA2 were used to amplify the 425 bp fragment from different *Pseudomonas* spp. strains. Amplifications were performed in 25 μl reaction mixtures containing 3 μl of diluted, heat-lysed cell suspension, 1 × PCR buffer (Perkin-Elmer, Nieuwerkerk aan de IJssel, the Netherlands), 1.5 mM $MgCl_2$ (Perkin Elmer), 200 μM of each, dATP, dCTP, dGTP, and dTTP (Promega, Leiden, the Netherlands), 20 pmol of each primer, and 2 U of AmpliTaq polymerase (Perkin-Elmer). The PCR program for *gacA* consisted of an initial denaturation at 95°C for 2 min followed by 30 cycles at 95°C for 1 min, annealing at 61°C for 1 min, and extension at 72°C for 1 min. Amplifications were carried out in a MJ Research PTC-200 thermocycler.

Sequencing of the gacA *gene*

Amplifications of the *gacA* gene with primers gacA1 and gacA2 were performed with the Expand High Fidelity PCR system, following the supplier's recommendations (Roche Diagnostics GmbH, Mannheim, Germany). Polymerase chain reaction products were run in a 1% agarose gel, bands were excised from the gel and purified by using the PCR purification kit (Qiagen, Hilden, Germany). This purification was performed to remove primer dimers and other residues from the PCR amplification. These fragments were sequenced with primers gacA1 and gacA2 using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) on an ABI sequencer.

Sequencing of 16S rDNA

Five microliters of the cell lysis suspension was used in 16S rDNA sequencing reactions. The primers used for sequencing were 8f and 1406r (Amann *et al*., 1995) and reaction conditions were as described by Liu *et al*. (1997). The PCR-amplified products were analysed by electrophoresis on 2% agarose gels. Fragments were cloned into the T/A vector pCR2.1 (Invitrogen, Carlsbad, CA), and transformed into *E. coli* strain INVαF′. Transformants were selected on LB agar amended with kanamycin (75 μg ml^{-1}) and X-Gal (20 μg ml^{-1}). Plasmid DNA was isolated from transformants using the S.N.A.P. mini-prep kit (Invitrogen). Cycle sequencing was conducted on a Perkin-Elmer model 480 thermal cycler (Norwalk, CT) and PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (PE Biosystems, Warrington, UK). Reactions were conducted using cycling conditions recommended by the manufacturer with the T3 or T7 primer. Products of the sequencing reactions were purified using CENTRI-SEP spin columns (Princeton Separations, Adelphia, NJ) and dried in a vacuum centrifuge. Samples were resuspended in 6 μl of a solution containing deionized formamide and blue dextran/EDTA in a ratio of 5 : 1. Samples were heated for

2 min at 90°C and 1.5 μl was loaded on an ABI 377 DNA sequencer (PE Biosystems, Foster City, CA). BLASTN searches were conducted on the nucleotide sequences obtained.

Nucleotide sequence accession number of gacA *and 16S rDNA*

Newly obtained sequences were deposited with GenBank under accession numbers described in Table 2.

Phylogenetic analysis

Prior to analysis, sequences of the *gacA* gene and its homologues were separated into the following categories: (1) all *gacA* sequences together (44 sequences); (2) *gacA* sequences from the Pseudomonadaceae and Enterobacteriaceae (36 sequences); (3) only *gacA* sequences from Pseudomonadaceae (22 sequences); and (4) only *gacA* sequences from Enterobacteriaceae (14 sequences). Amino acid and nucleotide sequences were aligned by using the CLUSTAL W 1.81 program (Thompson *et al*., 1994). Pairwise non-synonymous (Ka) and synonymous (Ks) nucleotide substitutions in the *gacA* sequences were calculated according to the Nei-Gojobori's method (Nei and Gojobori, 1986) as implemented in MEGA (Kumar *et al*., 2001). Fisher's exact test and *Z*-test for selection based on codon usage were performed by the Nei-Gojobori's method with the number of synonymous differences per synonymous site and the number of non-synonymous differences per non-synonymous site. Significance values for the *Z*-test were determined by using 1000 bootstrap replicates.

Majority sequences of *gacA* of species belonging to the Pseudomonaceae and Enterobacteriaceae were computed using Megalign (DNAstar, WI, USA) to reduce the number of two-by-two comparisons by the Tajima's test. Tajima's 1D relative rate test (Tajima, 1993) was used to estimate pairwise rate variation between majority sequences of *gacA* with the closest relative as outgroup. Phylogenetic trees of *gacA* and 16S rDNA were constructed by the PHYLIP 3.2 Package (Felsenstein, 1989), MEGA, and TREECON (Van de Peer and De Wachter, 1994) using the Neighbour-joining method (Saitou and Nei, 1987) with the nucleotide substitution model of Jukes and Cantor (1969). For comparison purposes, similar analyses were performed with the maximum likelihood method. Phylogenetic trees with the GacA protein sequences were constructed using the Neighbour-joining method with the PAM distance matrix of Dayhoff and were computed with the program PROTDIST of PHYLIP. Confidence of the tree topology was performed by bootstrap analysis on 1000 resamplings (Felsenstein, 1985).

Southern and colony hybridizations

DNA transfer from agarose gels and bacterial colony transfer to Hybond N^+ nylon membranes (Amersham Pharmacia Biotech) were performed according to standard methods (Sambrook and Russel, 2001). Membrane washes and hybridizations were performed following standard procedures (Sambrook and Russel, 2001). High-stringent conditions comprised prehybridization for 1.5 h at 65°C, hybridization for 12 h at 65°C, membrane washings twice each for 5 min with 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS at room temperature followed by membrane washings twice each for 30 min with 0.1 × SSC-0.1% SDS at 65°C. Low stringent conditions consisted of prehybridization and hybridization at 55°C and two membrane washings with 0.5 × SSC at 55°C. The gacA probe was generated by direct labelling in PCR using the DIG labelling mix (Roche, Almere, the Netherlands). Hybridized probes were immunodetected according to the protocols provided by the supplier.

Soils

Five soils were obtained from agricultural fields in the Netherlands. All soils were collected from the upper 50 cm of the soil profile, air dried for a week, and passed through a 0.5 cm mesh screen before use. Soils CB, SU, SV and SSB were collected in December 1997 from a polder in the South-West of the Netherlands, 10 km from the city of Bergen op Zoom. These soils are physicochemically very similar, containing on average, 27% clay, 10% silt and 51% sand. The organic matter content is on average 4.3% and the pH, determined after extraction with $CaCl_2$ is 7.5. Soil SL was collected in June 1998 from a polder in the North-East of the Netherlands, at Lovinkhoeve, located 30 km from the city of Lelystad. SL soil contains 21% clay, 12% silt, 54% sand, 3.6% organic matter, and pH, determined after extraction with $CaCl_2$ was 7.5.

Plant cultivation

Pots containing 200 g of sieved soil were sown with 15 seeds of wheat, cv. Bussard. Plants were grown in a climate chamber at 15°C with a 12 h photoperiod. Plants received 50 ml of one-third strength Hoagland's solution (macroelements only) (Zekri, 1995) twice a week. After 30 days of plant growth, the roots were harvested and used for isolation of root-associated bacteria.

Isolation of bacteria from the wheat rhizosphere

Three to five plants of each replicate were randomly harvested and loosely adhering soil was removed. Root samples of 0.2–1.0 gram were vortexed for 1 min, sonicated for 1 min in a ultrasonic cleaner, and dilution plated onto KMB^+. Plates were incubated at 25°C for 48 h. Population densities of bacteria harbouring *gacA* homologues were determined by colony hybridization with the gacA probe followed by confirmation by PCR with the gacA primers. Colonies that reacted positively in hybridization with the gacA probe and primers (*gacA*$^+$ isolates) were isolated and purified for further analysis; several colonies that reacted negatively in hybridization and subsequent PCR with the gacA probe and primers (*gacA*$^-$ isolates) were also included.

Characterization of rhizosphere bacteria harbouring gacA *homologues*

Isolates obtained from the rhizosphere of wheat were genotypically identified and characterized for the production of

specific extracellular enzymes and secondary metabolites known to be regulated by *gacA*. The first genotypic identification of the isolates was by PCR analysis with primers specific for the 16S rDNA of the genus *Pseudomonas (sensu stricto*) (Widmer *et al*., 1998). Secondly, isolates were clustered in genotypic groups by RAPD analysis with primers M13, M12 and D7 (Keel *et al*., 1996). The presence or absence of fragments generated by RAPD analysis (1 for the presence or 0 for the absence of a particular sized band in the gel) was used to calculate the pairwise coefficients of similarity (Nei/Li distances). Random amplified polymorphic DNA analysis was performed three times for each primer. Gas chromatograph-fatty acid methyl ester (GC-FAME) analysis was performed to identify *gacA*$^{-}$ bacterial isolates. For GC-FAME analysis, isolates were cultivated on tryptic soy broth agar (Becton Dickinson, Cockeysville, MD) and incubated for 24 h at 28°C. Cells were collected with a 4 mm diameter transfer loop and processed for extraction of fatty acids using the procedures as outlined by the manufacturer (Microbial ID, Newark, DE). Fatty acid methyl esters were analysed using a Microbial Identification System equipped with an HP5890 series II gas chromatograph, HP3365 Chem Station and version 3.9 of the aerobe library (Microbial ID).

For all isolates, presence of genes involved in the biosynthesis of specific antibiotics was performed with primers Phl2a and Phl2b for 2,4-diacetylphloroglucinol [2,4-DAPG] (Raaijmakers *et al*., 1997) and primers PHZ1 (5′-GGCGA CATGGTCAACGG-3′) and PHZ2 (5′-CGGCTGGCGGCG TATTC-3′) for phenazine antibiotics (D.V. Mavrodi, D.M. Weller and L.S. Thomashow, unpubl. data). Pyrrolnitrin and pyoluteorin were detected as previously described (Keel *et al*., 1996; Duffy and Défago, 1999). Protease, phospholipase C, chitinase activities were detected by growing the bacterial isolates on skim milk agar, egg yolk agar (Sacherer *et al*., 1994) and chitin-agar (De Boer *et al*., 1998) respectively. Production of biosurfactants and fluorescence of the isolated bacteria was determined by growth on medium containing CTAB-methylene blue (Siegmund and Wagner, 1991) and *Pseudomonas* medium F (Cho and Tiedje, 2000) respectively.

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